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Update on Mineral Nutrition

Iron: Nutritious, Noxious, and Not Readily Available[1]

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Fe(II) and Fe(III) are relatively small ions with a marked propensity to form six-coordinate complexes with ligands containing O, N, and S. This property, combined with the remarkable range of redox potentials covered by iron-containing enzymes, accounts for the role of iron in such fundamental reactions as ribonucleotide and dinitrogen reduction as well as in the energy-yielding electron transfer reactions of respiration and photosynthesis. At the same time, the chemical properties of iron place limitations on the cellular accumulation of this element. First, Fe(II) and Fe(III) can act catalytically to generate hydroxyl radicals that are the most potent oxidizing agents known (Table I). Because of the potential of iron for wreaking cellular havoc, organisms generally regulate its uptake; as well, they store iron in the form of ferritin, a multimeric protein consisting of a 24-subunit shell that can house up to 4500 atoms of iron in its central cavity (Theil, 1987). Iron stored in this manner is nontoxic and is readily available to the cell.

The second limit to iron acquisition is the fact that iron is found in nature mostly as a constituent of insoluble oxyhydroxide polymers of the general composition FeOOH (Table I). These Fe(III) oxides (e.g. goethite, hematite) are produced by the weathering of rock. Because Fe(III) oxides are quite stable and not very soluble at neutral pH, free Fe(III) in an aerobic, aqueous environment is limited to an equilibrium concentration of approximately 10^{-17} M, a value far below that required for the optimal growth of plants or microbes (Table I). Thus, the problem that soil-based organisms have with iron is not one of abundance, since iron ranks fourth among all elements on the surface of the earth, but rather one of availability in aerobic environments at biological pH. Iron deficiency can be particularly pronounced in plants grown on calcareous soils, which cover approximately one-third of the earth's surface. Iron deficiency is usually recognized by chlorotic or yellowed interveinal areas in new leaves and, if severe, can lead to reduction in crop yields and even complete crop failure.

Chemically speaking, organisms have three means at their disposal to dissolve Fe(III) oxides: protonation, chelation, and reduction. To compete successfully for iron, organisms have thus evolved specific mechanisms to acquire iron that are based on these chemical processes. In many organisms, including dicots, nongraminaceous monocots, and yeast, Fe(III) is initially solubilized by reduction and Fe(II) is then transported across the PM. In other organisms, including graminaceous monocots, bacteria, and fungi, Fe(III) is solubilized by being bound to high-affinity chelators called siderophores (or, in the case of plants, phytosiderophores). These Fe(III) complexes are then recognized by specific membrane receptors and iron transport ensues.

STRATEGY I: DICOTS AND NONGRAMINACEOUS MONOCOTS

Strategy I-type plants respond to iron deprivation with both morphological and physiological changes (Römheld, 1987). Morphological modifications include enhanced development of lateral roots and differentiation of specialized transfer cells. Both of these changes increase the surface area for reduction and transport of iron. Physiological responses covering all three means of accessing the iron tied up in Fe(III) oxides include increased acidification of the rhizosphere via enhanced proton extrusion and secretion of phenolics and organic acids to chelate iron, and reduction of Fe(III) to Fe(II) via an inducible, PM-bound Fe(III) reductase (Fig. 1).

Rhizosphere Acidification

Iron deficiency causes plant roots to extrude protons, which lowers the rhizosphere pH and solubilizes iron (Table I). The rate of proton excretion can be quite fast; within a few hours the roots may lower the pH in the soil solution to values of 3 or lower (Bienfait, 1985). In fact, the order for agents that can cause proton release by cells is iron deficiency > NH_4^+ > K^+ > fusicoccin (Bienfait, 1985). Presumably, a proton-pumping ATPase is activated. The capacity of a plant to acidify the rhizosphere in response to iron deficiency, of course, depends to some extent on the cation/anion uptake balance and the N nutrition of the plant.

Fe(III) Reduction

Enhanced Fe(III) reduction under iron deficiency is the most typical feature of strategy I and is probably the primary factor in making iron available for absorption. In soybean, Fe(III) reduction is an obligatory step in iron uptake; this is probably true for all strategy I plants (Römheld, 1987). Ferric reduction takes place at the PM of root epidermal cells via a

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Abbreviations: NA, nicotianamine; PM, plasma membrane.

Table I. *Iron-clad information*

Toxicity of Iron:

The Haber Weiss reaction (Eq. 3) is a sum of the reduction of Fe^{3+} by superoxide ion (Eq. 1) and the well-known Fenton Reaction (Eq. 2):

$$\cdot O_2^- + Fe^{3+} = O_2 + Fe^{2+} \quad (1)$$

$$H_2O_2 + Fe^{2+} = Fe^{3+} + OH^- + HO\cdot \quad (2)$$

$$\cdot O_2^- + H_2O_2 = O_2 + OH^- + HO\cdot \quad (3)$$

Solubility of Iron:

$Fe^{3+} + 3OH^- \rightarrow Fe(OH)_3$

$K_{sp}Fe(OH)_3 = [Fe^{3+}][OH^-]^3$

$K_{sp} = 10^{-38}$ M

$[Fe^{3+}] = 10^{-38}/[OH^-]^3 = 10^{-38}/[10^{-7}]^3 = 10^{-17}$ M (at pH 7.0)

Stability Constants (at pH 7.0) of Siderophores Commonly Found in Soil:

Ferrichrome, made by numerous fungi	10^{25}
Ferrioxamine, made by actinomycetes	10^{27}
Pseudobactin (pyoverdin), made by pseudomonads	10^{25}
Mugineic acid, a phytosiderophore	10^{17}

Iron Requirements:

Amount of iron required for optimal growth of plants: 10^{-9} to 10^{-4} M

Amount of iron in typical plant leaf: 50 to 100 μg/g dry weight

Amount of iron required for optimal growth of microbes: 10^{-7} to 10^{-5} M

transmembrane redox system that can be activated when iron is in short supply (e.g. Buckhout et al., 1989). Bienfait (1985) proposed that roots contain two Fe(III) reductase activities: one that is capable of reducing Fe(III) chelates and ferricyanide, and another that can only reduce ferricyanide. The inducible Fe(III) chelate reductase system (termed the turbo reductase) was thought to be expressed in the epidermal cells of young, lateral roots grown under iron deficiency, whereas the ferricyanide reductase (termed the standard reductase) was thought to be constitutively expressed in all root cells.

However, more recent studies do not support this view. Both ferricyanide and Fe(III) chelate reductases were found to increase approximately 2-fold under iron-deficiency conditions in PM purified from tomato (Buckhout et al., 1989). PM from roots of iron-sufficient and iron-deficient plants contain Fe(III) chelate reductases with similar characteristics (Holden et al., 1991), thus lending no support to the concept of the induction of a novel reductase in response to iron deficiency. The amplification of Fe(III) chelate reductase observed in PM isolated from iron-deficient plants likely involves increased expression of Fe(III) chelate reductase isoforms in expanding root epidermal PM. Thus, the Fe(III) chelate reductase either is being activated or is being synthesized in increased amounts. PM isolated from roots of iron-deficient plants contained 2- to 3-fold higher specific activities for Fe(III) chelate reductase than PM isolated from plants grown under iron-sufficient conditions (Buckhout et al., 1989). Partial purification of the Fe(III) chelate reductase from tomato has implicated a 35-kD polypeptide as a putative component of the reductase (Holden et al., 1994).

What is the substrate for Fe(III) reductase in situ? It seems that the various microbial siderophores can serve as substrates for Fe(III) reductase. However, exact estimates of the abundance of siderophores in the rhizosphere are hard to come by. Several monoclonal antibodies raised against various siderophores should help in determining siderophore levels and the distribution of siderophores in soil (e.g. Buyer et al., 1993). Siderophores are produced by a wide range of soil microorganisms. In addition to being a factor that may influence the iron nutrition of plants, microbial siderophores have been implicated as determinants of biocontrol activity and as virulence factors in plant disease (Loper and Buyer, 1991). At present, chrysobactin produced by *Erwinia chrysanthemi*, a bacterial pathogen causing soft rot on a wide range

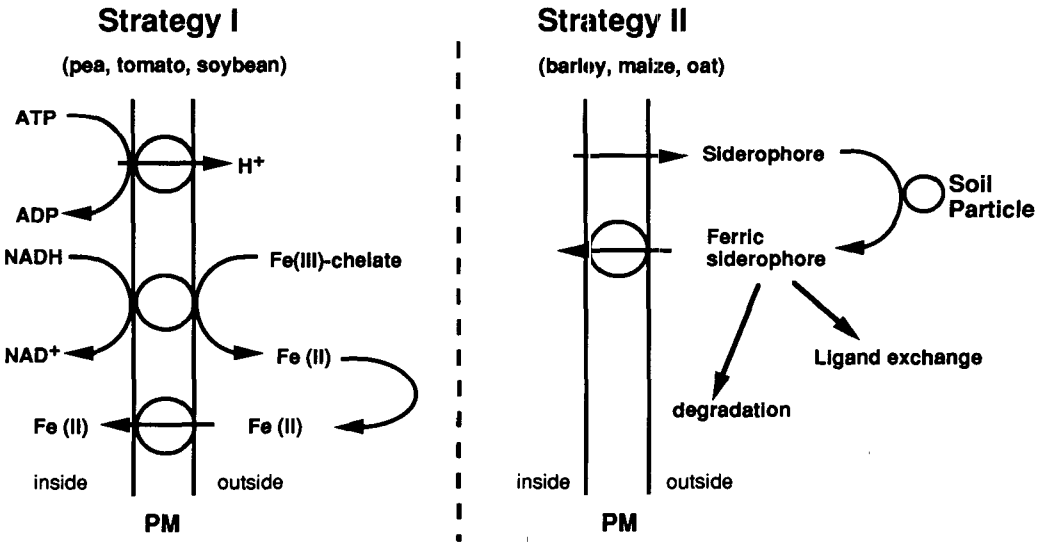

Figure 1. Models for iron acquisition by higher plants. Only the best-studied features are shown.

of plants, is the only siderophore shown to be a virulence factor in plant disease.

In addition to its role in reducing Fe(III) chelates, Fe(III) reductase may play a more general role in regulating cation uptake. Welch et al. (1993) have recently suggested that root PM reductases may control the reduction of critical sulfhydryls in transport proteins involved in divalent cation transport across the root PM. Roots from both iron-deficient and copper-deficient plants reduced exogenous Cu(II) chelate as well as Fe(III) chelate. When reductase activity was stimulated by iron deficiency, the absorption of a number of cations increased. Evans et al. (1992) have also reported that roots grown under low-iron conditions accumulated twice as much copper as roots grown under conditions of high available iron.

IS YEAST A GOOD MODEL FOR THE STRATEGY I RESPONSE OF PLANTS?

The current model for the mechanism of iron uptake in yeast is that a PM Fe(III) reductase reduces Fe(III) to Fe(II), which then crosses the membrane via an Fe(II)-specific transport system. A yeast mutant, *fre1-1*, had the phenotypes predicted by the model; *fre1-1* lacked plasma membrane Fe(III) reductase activity and was also deficient in the uptake of Fe(III), but not in the uptake of Fe(II) (Dancis et al., 1990). A mutant such as *fre1-1*, which cannot reduce iron, would not produce any ferrous iron for transport. Sequence analysis of the yeast *FRE1* gene (which was identified by complementation of the *fre1-1* mutant) revealed a single long open reading frame of 686 amino acids with a predicted molecular mass of 78.8 kD (Dancis et al., 1992). The open reading frame has limited homology (17.9% identity and 62.2% similarity over the carboxy-terminal 402 amino acids of FRE1) to the large subunit of human Cyt b_{558} (gp91-*phox*), the protein affected in the X-linked form of chronic granulomatous disease (Dancis et al., 1992). Cyt b_{558} is a component of the respiratory burst oxidase of neutrophils, a membrane-bound enzyme complex that catalyzes the one-electron reduction of oxygen to O_2^-. Because the respiratory burst oxidase is involved in the movement of a single electron from NADPH across the PM to molecular oxygen, the similarity to FRE1 may be significant; the yeast Fe(III) reductase must move single electrons across the PM as well, although in the case of Fe(III) reductase, the terminal electron acceptor is Fe(III). The yeast Fe(III) reductase and the human granulocyte reductase are most similar in regions of the protein thought to be involved in the binding of flavin adenine dinucleotide and NADPH (Roman et al., 1993). These regions are also conserved with the *Schizosaccharomyces pombe* Frp1 protein. The *Frp1* gene, like the *FRE1* gene of yeast, was identified by complementation of an *S. pombe* Fe(III) reductase mutant (Roman et al., 1993). It has been suggested that FRE1, Frp1, and gp91-*phox* are members of a novel family of PM electron transport proteins responsible for mobilizing cytoplasmic reducing equivalents and donating them to extracellular substrates (Roman et al., 1993). The degree of amino acid identity between the *S. pombe* Frp1 and the *Saccharomyces cerevisiae* FRE1 is quite limited (27% amino acid identity and 49% similarity). Thus, although similar iron uptake mechanisms are likely to operate in higher organisms, the responsible proteins may have little overall sequence conservation. We have identified three different *Arabidopsis* genomic clones that show homology to the FRE1 gene of yeast. One of the clones has an open reading frame that encodes a protein that has 17.5% identity and 48.9% similarity with the deduced FRE1 protein (Yi et al., 1994). Work is underway to determine whether the *Arabidopsis* clones encode proteins that have Fe(III) reductase activity.

So, is yeast a good model for iron uptake in plants? Yeast, like plants, reduces iron before transport via a PM-bound Fe(III) reductase. Yeast, like plants, appears to have an Fe(II) transporter. Eide et al. (1992) have provided convincing biochemical evidence that *S. cerevisiae* has a transporter that is specific for Fe(II) and whose activity can clearly be separated from that of the Fe(III) reductase. This is in contrast to earlier work that suggested, on the basis of inhibitor studies, that Fe(II) might be transported by the common divalent cation transporter that transports zinc, cobalt, and nickel. The observation that the yeast PM Fe(III) reductase is not only involved in reductive iron uptake but plays a more general role in modifying the redox potential of cells may also be true for plants (Lesuisse and Labbe, 1992; Welch et al., 1993). A comparison of information available on plants and yeast also reveals some differences. Unlike plants, there does not appear to be any involvement of intracellular citrate or malate in the trans-PM redox system of yeast, nor is there any substantial difference in the transmembrane electrical potential of iron-sufficient and iron-deficient cells of yeast (Lesuisse and Labbe, 1992). The yeast Fe(III) reductase utilizes NADPH, whereas the plant Fe(III) reductase preferentially utilizes NADH (Holden et al., 1994). Heme-deficient mutants of *S. cerevisiae* have both impaired Fe(III) reductase activity and impaired iron uptake. The role of heme in the Fe(III) reductase system of *S. cerevisiae* remains unclear, although most membrane electron transport systems, including the respiratory burst oxidase of human phagocytes, have at least one heme-containing subunit. At present there is no evidence of a heme-containing component in the iron-chelate reductase from tomato (Holden et al., 1994).

STRATEGY II: GRAMINACEOUS MONOCOTS

Strategy II plants are characterized by the release of phytosiderophores (e.g. mugineic acid in barley and avenic acid in oat) and by the induction of a high-affinity uptake system for Fe(III) phytosiderophores (Fig. 1) that transports the Fe(III) chelates as intact molecules (Römheld, 1987). This strategy is considered to be more efficient than strategy I; for example, grasses can grow on calcareous soils that will not support the growth of dicots. One reason for this may be that strategy II is less pH dependent than strategy I. The phytosiderophores that have been characterized are structurally related to NA, a nonprotein amino acid that is thought to be an iron carrier in plants (Stephan and Scholz, 1993). Indeed, there is evidence that NA is an intermediate in the formation of the mugineic acid family of phytosiderophores.

There are a number of considerations when trying to evaluate the ecological importance of phytosiderophores. First, what are concentrations of phytosiderophores in the

rhizosphere? Second, do rhizosphere microorganisms affect the distribution of phytosiderophores? Third, how successfully do phytosiderophores compete with microbial siderophores for iron? Fourth, do phytosiderophores mobilize other elements besides iron?

Briefly, plants that have been appropriately examined release appreciable amounts of phytosiderophores with a distinct diurnal rhythm to the release (Römheld, 1991). It has been suggested that the release of a single large quantity once a day may increase the probability that phytosiderophores will mobilize iron before being degraded by rhizosphere microoganisms. As might be expected, there are a number of bacteria that can utilize phytosiderophores as a sole source of carbon as well as a source of iron. In at least one case, utilization of phytosiderophores as an iron source appears to be indirect, with ligand exchange occurring between the microbial siderophore and the phytosiderophores (Jurkevitch et al., 1993). It has been suggested that physical separation on the root between zones of active release and uptake of phytosiderophores and heavy colonization by bacteria protect phytosiderophores not only from degradation but also from competition for iron with bacterial siderophores (Römheld, 1991).

In general, microbial siderophores have a higher affinity for iron than phytosiderophores, although the stability constants that have been calculated for phytosiderophores are believed to be far too low (Table I). Attempts to determine whether strategy II plants can directly utilize microbial siderophores have yielded conflicting data, in large part due to the use of nonaxenic conditions. Experiments that take into account the effects of rhizosphere microoganisms suggest that microbial siderophores do not serve directly as sources of iron for strategy II plants (Bar-Ness et al., 1992). However, they may serve indirectly, after degradation by microbes and release of iron, which can then complex with phytosiderophores.

Efforts are underway to isolate and characterize the genes involved in the acquisition of iron by Gramineae. In barley, a differential hybridization approach has identified cDNAs specific to iron deficiency; some of these genes may encode products that are involved in the synthesis, secretion, or uptake of the barley phytosiderophore mugineic acid (Nakanishi et al., 1993). Although *Ids1*, the first cDNA clone characterized, was found to encode a protein that is homologous to metallothionein, two of the other clones identified (*Ids2* and *Ids3*) encode proteins that show homology to 2-oxoglutarate-dependent dioxygenase, suggesting that they may function in the hydroxylation process leading to the synthesis of mugineic acid.

WHAT HAVE WE LEARNED FROM PLANT MUTANTS WITH ALTERED IRON UPTAKE PHENOTYPES?

There are a number of plant mutants that have been described to date that have increased or decreased rates of iron uptake. The *chloronerva* mutant of tomato accumulates iron, as does the *brz* mutant of pea (Welch and Kochian, 1992; Stephan and Scholz, 1993). The *chloronerva* mutant is an NA auxotroph; application of NA to the roots or leaves of mutant plants leads to recovery (Stephan and Scholz, 1993). The link between the lack of NA and increased iron uptake is still unknown. Because NA is a chelator for Fe(II) at physiological pH, it has been proposed that iron bound to NA may be the "active" form of iron that binds to an iron sensor or gene regulator. Thus, the mutant that has no NA suffers from apparent iron deficiency and fails to repress inducible iron-uptake processes. A role for NA as a mediator of iron transport in the phloem could also explain the various phenotypes of the *chloronerva* mutant (Stephan and Scholz, 1993). The *brz* mutant of pea develops bronze necrotic spots on its leaves and contains 50-fold more iron in its leaves than do leaves from wild-type plants. The basis for the excess iron accumulation appears to be higher rates of Fe(III) reduction; *brz* plants had high rates of Fe(III) reduction regardless of plant iron status. There is no increase in the activity of the iron transport system in the *brz* mutant. In fact, it does not appear that plants induce the synthesis of a transport protein in response to iron deficiency, although many microorganisms are known to do so (Welch and Kochian, 1992). The *brz* mutant also accumulates high levels of other divalent cations [Mg(II), Mn(II), Zn(II)], which is consistent with a role for Fe(III) reductase in general cation uptake, as has been suggested by Welch et al. (1994). Unlike the *chloronerva* and *brz* mutants, which take up too much iron regardless of iron status, the *fer* mutant of tomato is unable to respond to iron deficiency, suggesting that FER encodes a positive activator that regulates proteins such as Fe(III) reductase (Bienfait, 1988). At least two membrane proteins that are produced under iron-deficient conditions are not produced in the *fer* mutant. Finally, the yellow stripe mutant of maize, *ys1*, is unable to respond to iron deficiency and is currently under investigation in several laboratories as a means of understanding strategy II responses.

Given the often-cited advantages of using *Arabidopsis* as a model system (Meyerowitz, 1989), we wanted to use *Arabidopsis* to study iron uptake in plants. *Arabidopsis* exhibits a strategy I-type response, showing increased Fe(III) reductase activity under iron deficiency as well as increased acidification of the rhizosphere (Yi et al., 1994). We have been screening mutagenized populations of *Arabidopsis* for mutants that have either a decreased or an increased capacity to acquire iron relative to wild type, looking in particular for mutants in Fe(III) reductase activity. Using an assay based on the affinity of the dye FerroZine to form a colored complex with Fe(II), we have identified a number of *Arabidopsis* mutants that do not show elevated levels of Fe(III) reductase activity under iron-deficient growth conditions (Yi et al., 1994). These mutants may either: (a) have defects in Fe(III) reductase itself, (b) be defective for a gene that controls Fe(III) reductase, or (c) exhibit some type of root defect that prevents proper expression of Fe(III) reductase.

FUTURE DIRECTIONS

Little attention has gone into the study of the movement of iron after it crosses the root PM. We need to investigate the movement to and loading of the xylem vessel in the root stele. Once it is in the xylem, iron is believed to be transported as ferric citrate. However, this needs to be examined in more species. We need to understand how iron is unloaded from

the xylem into the apoplastic spaces of leaf mesophyll cells and then how leaf cells absorb iron. Uptake of Fe(III) by leaf mesophyll appears to depend on a reduction step via a PM-bound Fe(III) chelate reductase, which most likely releases Fe(II) from Fe(III) citrate (Brüggemann et al., 1993). There is also some evidence that Fe(III) reduction in vivo may be aided by intermediate superoxide radical formation or by strong blue light (Brüggemann et al., 1993). We know very little about the movement of iron in the phloem sap. As mentioned previously, NA may act to transport iron in the phloem. One of the most important steps to understand is the deposition of iron in seed and grain tissues via the phloem. We also need to know more about the bioavailability of iron, which is deposited in the edible portions of plants such as seeds and grains. Finally, we need to know more about how plants acquire iron normally, i.e. when they are not growing under iron-deficient conditions. Grusak (1993) has followed Fe(III) chelate reductase activity over the time course of development in pea and found an increase in Fe(III) chelate reductase activity during pod fill, when there is an increased demand for iron.

To avoid the charge of being terrestriocentric, we must include a few words about iron and its availability in aquatic environments. Aquatic organisms are faced with the same problems as terrestrial organisms regarding the solubility of iron. Several years ago it was suggested that a quick fix to the greenhouse problem would be to dump iron into the Southern Ocean near Antarctica. Termed the "Geritol solution," it was based on the hypothesis that Antarctic phytoplankton suffer from iron deficiency, which prevents them from blooming and using up the otherwise abundant supply of other nutrients found in the Southern Ocean (Martin et al., 1990). Not only is it unclear at present whether addition of iron would stimulate phytoplankton productivity, it is even less clear how much carbon dioxide would be absorbed (Geider and La Roche, 1994). The minimum amount of iron required for maximum growth of oceanic phytoplankton appears to be much less than one would predict is needed for photosynthesis, respiration, and nitrate reduction. One explanation for this discrepancy may be the substitution of flavodoxins for Fds when iron is limiting; such an adaptation has now been documented in both cyanobacteria (Fillat et al., 1988) and phytoplankton (La Roche et al., 1993).

CONCLUDING REMARKS

Iron deficiency in humans is the most prevalent nutritional problem in the world today (Scrimshaw, 1991). Although vegetables, particularly spinach, are regarded as impressive sources of iron, plant (nonheme) iron is poorly absorbed; only 1.4% of the iron from spinach can be taken into the body, compared with 20% from red meat (Scrimshaw, 1991). To have a significant impact on the iron nutrition of humans, plant scientists need to develop and introduce new varieties of major seed and grain crops that contain significantly increased amounts of bioavailable iron. It will probably not be sufficient to merely increase iron uptake by plants, but this is certainly a good starting point, especially in areas of the world where the availability of soil iron limits crop yields. The study of iron uptake in plants should ultimately lead to the construction of iron-efficient plant varieties and will have application to general transmembrane permeation processes and their regulation.

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